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DRG: A NOVEL DEVELOPMENTALLY REGULATED GTP-BINDING PROTEIN

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SUMMARY: Using a subtraction cloning approach we had previously isolated a series of murine cDNA clones representing the genes predominantly expressed in the embryonic brain and down-regulated during development. We now report that one of these cDNA clones encodes a novel type of GTP-binding protein. The predicted protein of 40.5 kD, named DRG, contains five structural motifs characteristic of the GTP-binding proteins. Consistently, bacterially expressed and cellular DRG proteins are capable of binding GTP *in vitro*. Sequences closely related to the DRG protein are found in other species including *Drosophila* and *Halobacterium*. Based on these observations, we propose that DRG respresents an evolutionarily conserved novel class of GTP-binding protein which may play an important role in cell physiology.

We are interested in identifying genes which control the early development and differentiation of mouse central nervous system (CNS) precursor cells, and to this end we have tried to isolate cDNA clones for mRNA species which are predominantly expressed during the early stages of CNS development. This was achieved by using a cDNA library prepared from CNS precursor cells and subtracted with excess RNA from post-natal and adult brain (1). Using this strategy, ten independent and novel cDNA clones were isolated whose corresponding genes showed down-regulation of expression in brain during development (1). As these genes are expressed at much higher levels in the developing and differentiating brain, than the fully differentiated adult brain, they are likely to play a role in early neurogenesis. In the present paper we describe the characterisation of one such gene *NEDD-3*, which encodes a novel GTP-binding protein. We have renamed the *NEDD-3* gene as *drg* and its product DRG, for Developmentally Regulated GTP-binding (gene/ protein).

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Abbreviations: CNS, central nervous system; E(x), post-coital embryonic day (x); NPC, neural precursor cells; MBP, maltose-binding protein; GST, glutathione S-transferase.

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EXPERIMENTAL PROCEDURES

Cloning of drg *cDNA*

Clone 2A7 was isolated from a neural precursor cell (NPC) directional cDNA library in λ uni ZAP vector (Stratagene) which had been subtracted with a large excess of mRNA isolated from post-natal and adult brain (1). This clone contained a 0.5 kb cDNA insert which was used as a probe to screen two cDNA libraries; the original NPC library and a commercially available random primed cDNA library from PCC4 cell line (Stratagene) according to standard protocols (2). Several hybridising clones (Figure 1) were purified by three cycles of screening. Some of the clones isolated from the PCC4 library had chimeric fusions with other cDNA and for this reason several clones were sequenced from Exo III nested deletions of the plasmid DNA. For the rescue of pBluescript (SK-) from the lambda Zap (Stratagene) clones, Exo III deletions (Pharmacia) and DNA sequencing (USB), protocols provided by the manufacturers were used. Some double-stranded DNA sequencing was also performed using Applied Biosystem 373A DNA sequencer. Computer analysis was performed by the Wisconsin Genetics Computer Group program package containing FASTA and TFASTA (3).

Expression of DRG Fusion Proteins in Bacteria and Inoculation of Rabbits

The open reading frame of the cDNA was amplified by PCR using upstream (5'-GGAGGC TGGTGTGGCGCTAGCCCCTCC-3') and downstream (5'-CTGTGGCCAGCTAGCAGA TGGCAAG-3') primers (bases modified from the original sequence to create a Nhe I site are underlined). The PCR-generated fragment was digested with Nhe I and cloned into the Xba I site of a maltose-binding protein (MBP) fusion vector pMal-c (4,5) purchased from New England Biolabs. For cloning into the glutathione S-transferase (GST) fusion vector (6), a Bam HI and Eco RV fragment derived from clone 2A725 (Figure 1A) was ligated into the Bam HI/ Sma I sites of pGEX-2T (Pharmacia). The estimated sizes of the MBP- and GST-DRG fusion protein are 82.5kD and 66.5kD respectively. Fusion proteins were purified by affinity chromatography according to the instructions supplied by the manufacturers. Antiserum against MBP-DRG fusion protein was raised in rabbits following standard protocols (7). Four hundred μg of the fusion protein with Freund's complete adjuvant were inoculated subcutaneously at multiple sites. Boosters, at monthly intervals consisted of 250 μg of the fusion protein in Freund's incomplete adjuvant introduced intra-muscularly. Rabbits were bled 6-10 days post-booster and antibody titres were determined by immuno blotting. A 1: 2000 dilution of the serum could detect 1.25 μg of the fusion protein in immuno blot analysis.

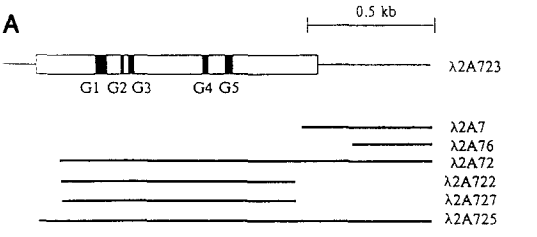
GTP-Binding Assays

GTP-binding assays were performed essentially as described (8). Briefly, E13 brain tissue was homogenised in TNE buffer (10mM Tris-Cl, pH 7.8, 1% NP 40, 150mM NaCl, 1mM EDTA, and 10 μg/ ml aprotinin) on ice, centrifuged and supernatant dialysed against several changes of phosphate buffered saline (PBS). Ten μg of E13 brain extract or 1μg of fusion protein were incubated with 10μCi [^{32}P]GTP in the binding buffer (20mM Tris.HCl, pH 8.0, 10mM EDTA, 5mM $MgCl_2$/ 2mM $MnCl_2$, 1mM DTT, 10μM ATP) for 2h at 30° C, and then irradiated on ice for 30 min at 5 cm from a UV source (Stratalinker, Stratagene). The samples were incubated with rabbit anti MBP-DRG serum for 2h, followed by 1h incubation with protein A-Sepharose on ice. Protein A-
antibody complexes were washed several times in TNE, boiled in 1x sample buffer and electrophoresed on SDS-polyacrylamide gels. Dried gels were exposed to imaging plates, analysed and printed using BAS2000 Bioimage Analyser.

RESULTS AND DISCUSSION

Cloning of drg *cDNA*

Using the original 0.5 kb *NEDD-3* cDNA clone (2A7), which respresents the 3'-end of the mRNA (1), two cDNA libraries, one from CNS precursor cells and the other from embryonic carcinoma cell line PCC4, were screened. A number of hybridisation positive clones were isolated and sequenced. The near full length cDNA sequence of 1560 bp (including the poly A tail) thus obtained is shown in Figure 1. The sequence contains a single long open reading frame which is continuous at its 5'-end. The first inframe ATG is located 76 bp downstream from the



B

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   1  GCCAGAGGCGCGGCGGGCGCGAGAAGGGGGACCTAACCAGTGTGGAGGCTGGTGTGGCCT   60
  61  TAAGCCCTCCGCCACCATGAGCGGGACCTTAGCGAAGATCGCGGAGATCGAAGCCGAGAT  120
                  M  S  G  T  L  A  K  I  A  E  I  E  A  E  M   15
 121  GGCTCGGACTCAGAAGAACAAAGCCACAGCTCATCACCTTGGCCTGCTTAAGGCTCGCCT  180
  16   A  R  T  Q  K  N  K  A  T  A  H  H  L  G  L  L  K  A  R  L   35
 181  TGCTAAACTTCGAAGAGAACTCATCACTCCTAAAGGTGGCGGCGGCGGGGGGCCAGGAGA  240
  36   A  K  L  R  R  E  L  I  T  P  K  G  G  G  G  G  G  P  G  E   55
 241  AGGTTTTGATGTGGCCAAGACAGGTGATGCTCGAATTGGGTTTGTGGGTTTTCCATCTGT  300
  56   G  F  D  V  A  K  T  G  D  A  R  I  G  F  V  G  F  P  S  V   75
 301  GGGGAAATCAACTCTGCTAAGTAACTTAGCAGGAGTGTATTCTGAAGTGGCAGCCTATGA  360
  76   G  K  S  T  L  L  S  N  L  A  G  V  Y  S  E  V  A  A  Y  E   95
 361  ATTCACTACATTGACCACAGTGCCTGGTGTCATCAGATATAAAGGTGCCAAGATCCAGCT  420
  96   F  T  T  L  T  T  V  P  G  V  I  R  Y  K  G  A  K  I  Q  L  115
 421  CCTGGATCTCCCGGGTATTATTGAAGGTGCCAAGGATGGGAAAGGTAGAGGTCGTCAAGT  480
 116   L  D  L  P  G  I  I  E  G  A  K  D  G  K  G  R  G  R  Q  V  135
 481  CATTGCAGTGGCCCGAACGTGTAATTTGATTTTGATTGTTCTGGATGTCCTAAAACCCTT  540
 136   I  A  V  A  R  T  C  N  L  I  L  I  V  L  D  V  L  K  P  L  155
 541  GGGACATAAGAAGATAATTGAAAATGAGTTGGAAGGCTTTGGGATTCGCTTGAACAGCAA  600
 156   G  H  K  K  I  I  E  N  E  L  E  G  F  G  I  R  L  N  S  K  175
 601  ACCCCCCAACATTGGCTTTAAGAAGAAAGACAAGGGAGGCATTAACCTCACAGCCACTTG  660
 176   P  P  N  I  G  F  K  K  K  D  K  G  G  I  N  L  T  A  T  C  195
 661  TGCTCAGAGTGAACTGGATGCTGAAACAGTGAAGAGCATTCTGGCTGAATATAAGATTCA  720
 196   P  Q  S  E  L  D  A  E  T  V  K  S  I  L  A  E  Y  K  I  H  215
 721  TAATGCTGATGTGACTTTGCGTAGTGATGCCACAGCTGACGAGCTCATTGACGTGGTCGA  780
 216   N  A  D  V  T  L  R  S  D  A  T  A  D  D  L  I  D  V  V  E  235
 781  AGGGAACAGAGTTTATATTCCCTGTATCTATGTATTAAAATAAGATTGATCAGATCTCCAT  840
 236   G  N  R  V  Y  I  P  C  I  Y  V  L  N  K  I  D  Q  I  S  I  255
 841  TGAGGAGTTGGATATTATCTATAAGGTGCCTCACTGTGTACCCATCTCTGCTCATCACCG  900
 256   E  E  L  D  I  I  Y  K  V  P  H  C  V  P  I  S  A  H  H  R  275
 901  CTGGAATTTTGATGACCTTTTGGAGAAAATCTGGGACTATCTGAAACTAGTGAGAATTTA  960
 276   W  N  F  D  D  L  L  E  K  I  W  D  Y  L  K  L  V  R  I  Y  295
 961  CACCAAACCGAAAGGCCAGTTACCAGATTACACATCTCCAGTAGTGCTGCCTTATTCTAG 1020
 296   T  K  P  K  G  Q  L  P  D  Y  T  S  P  V  V  L  P  Y  S  R  315
1021  GACCACTGTGGAAGATTTCTGCATGAAGATTCACAAAAATCTCATCAAAGAATTTAAATA 1080
 316   T  T  V  E  D  F  C  M  K  I  H  K  N  L  I  K  E  F  K  Y  335
1081  TGCTTTGGTCTGGGGTCTGTCTGTGAAACATAATCCTCAGAAAGTGGGTAAAGACCACAC 1140
 336   A  L  V  W  G  L  S  V  K  H  N  P  Q  K  V  G  K  D  H  T  355
1141  ATTGGAAGATGAAGACGTCATTCAGATTGTGAAGAAGTGAAGTCCTCTTGCCATCTGCTG 1200
 356   L  E  D  E  D  V  I  Q  I  V  K  K  *                      375
1201  GCTGGCCACAGCAGTGTTCCTTATGACTAAGTACCTTCCTCAGCCCCTCTGTCTTTGGCA 1260
1261  GCCTCTGGATTGGGATACAGGGATTGATATGGAGACTTATCAAACTGAAACACCACAATT 1320
1321  TTTTTCTTTCCGGAGATAGGGTGTTATGTATTTCAGGCTGGCTTTGAACTCTTGCAGCTG 1380
1381  AGGCTGGCCTAGAATCCCTTTCATCAGCCTCCATCTCCTCAGTGCTGGGCTTACCACTGT 1440
1441  GCACCTTTAGCTCCTCAGCAACACCACTTTCCCTACTTTGCTGTCACTTCTATTGAACTG 1500
1501  TATAAAGAAGCTGGTGGGCCCGCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1560
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Figure 1. Nucleotide and deduced amino acid sequence of *drg* cDNA.
(A) Physical maps of the cDNA clones isolated from the NPC and PCC4 cell cDNA libraries. A 0.5 kb insert derived from the original clone 2A7 (1) was used as a probe. The longest clone, 2A723, is shown in the upper panel. The location of the longest reading frame (starting at ATG) is shown as a box. The conserved GTP-interacting domains (11) are shown as black boxes.
(B) Complete nucleotide and predicted amino acid sequence of *drg* cDNA. The nucleotide sequence was derived from several clones shown in A. The GTP-interacting domains are boxed, the possible N-glycosylation site is encircled and the putative polyadenylation signal is underlined.

5'-end of the sequence. Although the reading frame is open at its 5'-end (i. e. the first ATG in the frame is not preceeded by a stop codon), it is likely that the cDNA sequence covers complete coding region of the *drg* mRNA, because the sequence of 1560 bp is close to the 1.6 kb size of the mRNA detected in the Northern analysis (1), and the first ATG in the frame is in a highly favorable context for mammalian translation initiation (9). The open reading frame ends with a TGA stop codon at the 1178th base. Downstream from the open reading frame is 347 bp of 3'-untranslated sequence, followed by a poly A tract. A possible polyadenylation signal TATAAA is located 18 bp upstream of the start of the poly A tract. The open reading frame of the cDNA

can potentially encode a protein of 367 amino acids with a predicted molecular weight of about 40.5 kD. There is one potential N-glycosylation site located at the amino acid residue 190.

The Putative Product of* drg *is a Evolutionarily Conserved Protein

Data base search using the FASTA program (3) resulted in the identification of a nucleotide sequence fragment sharing extensive homology (69.7% in 723 bp) with the *drg* cDNA sequence: a *Drosophila* genomic sequence located upstream of RNA polymerase III gene (EMBL acc. no. X58826) (Figure 2A). The homologous region is located in the region upstream of the gene of interest to the original authors. As we deduced the peptide sequences of the putative gene product, and aligned it with the predicted peptide sequence of the DRG protein, we found striking degree of homology (79.9% identity and 89.4% similarity in 273 amino acid residues) between the two putative proteins (Figure 2B). The data base entry contains incomplete coding region of the putative *Drosophila* protein. Subsequent correspondence with the authors indicated that the *Drosophila* locus upstream of the RNA polymerase III gene is indeed transcribed into a mRNA and a corresponding cDNA has now been cloned (G. Petersen, K. Sommer and E.K.F. Bautz, personal communication). The deduced full length protein sequences from *Drosophila* (G. Petersen, K. Sommer, and E.K.F. Bautz, personal communication) and *drg* cDNA show 79.8% identity and 88.3% similarity in 367 amino acid residues.

Search for DRG related peptides in the protein sequence databases, resulted in the identification of another peptide sequence: *Halobacterium* ORF putative protein (10), which showed 41.1% identity and 62.8% similarity in 372 amino acid residues, with predicted DRG protein. The *Halobacterium* ORF sequence is located in a chromosomal DNA fragment containing the L11e, L1e, L10e, and L12e ribosomal protein gene cluster (10). No biochemical or structural features of the putative ORF protein have been mentioned except that it shows no similarites to any known protein sequences and the gene is weakly transcribed (10). The alignment of DRG with putative *Drosophila* and *Halobacterium* protein sequences showed several regions of virtual identity, most notably in the NH_2-terminal 150 amino acid residues. Note that the *Drosophila* sequence shown in Figure 2B was derived from the database and lacks the first 94 residues at the NH_2-terminus, however the complete protein seqence derived from the cDNA (G. Petersen, K. Sommer, and E.K.F. Bautz, personal communication) also shows strong homology in the NH_2-terminus. Following this, the DRG and *Drosophila* sequences show strong identity, while the *Halobacterium* sequence shows identity in certain regions (Figure 2B). Based on these homologies and keeping in mind the evolutionary distances between mouse, *Drosophila* and *Halobacterium*, it is reasonable to assume that the *Drosophila* and *Halobacterium* genes are the *drg* counterparts of their respective host species.

drg *Encodes a Novel GTP-Binding Protein*

Local homology searches for sequences related to the putative DRG protein in the protein data base extracted a number of proteins all of which belonged to a single biochemical class of proteins, the GTP-binding proteins. The sequence similarities were limited to small regions which turned out to be the critical regions of GTP-binding proteins, termed G-1 to G-5, required for GDP/GTP-exchange, GTP-induced conformational changes and GTP hydrolysis (11). The

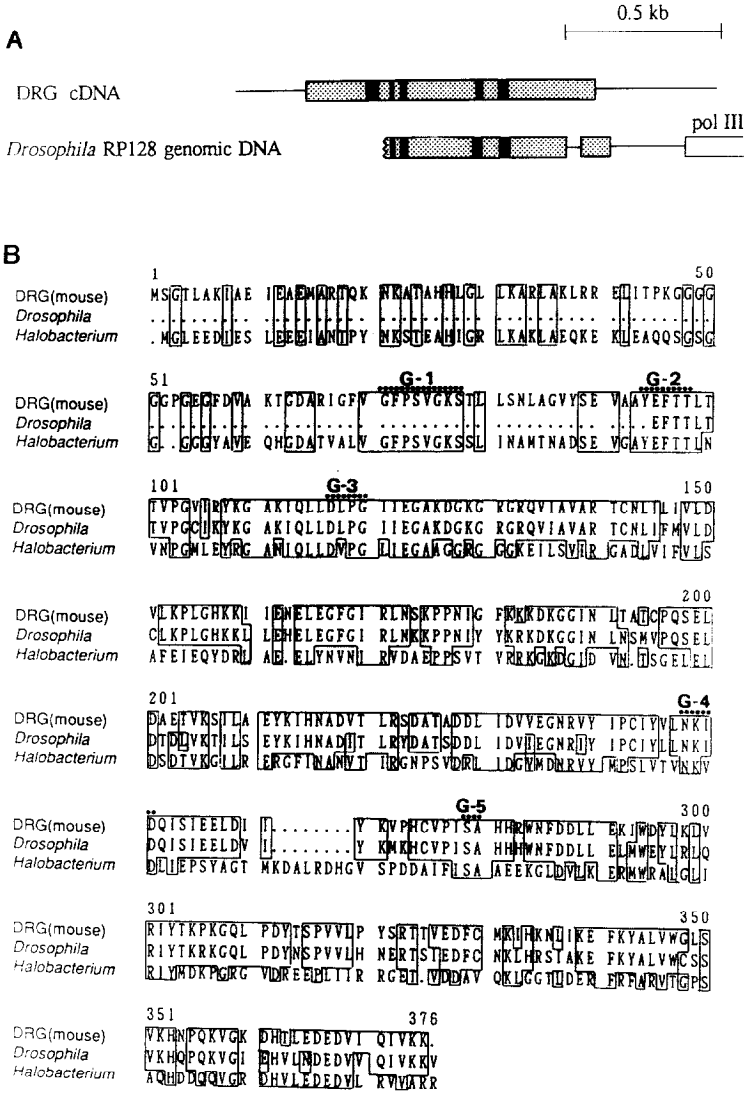

Figure 2. Homology of *drg* with sequences extracted from the databases.
(A) Structural similarities between the *drg* cDNA and the *Drosophila* genomic locus, RP128, upstream of RNA polymerase III gene (EMBL accession number X58826). Shaded boxes show the location of open reading frames and similarities between the two sequences. Note that following modification have been made to the data base entry (EMBL X58826) in order to bring the *Drosophila* frame in alignment with the mouse DRG: nucleotide A at position 441 deleted, single nucleotides G and A inserted after positions 551 and 606,respectively.
(B) Alignment of the mouse DRG amino acid sequence with the putative *Drosophila* protein translated from the genomic sequence upstream of RNA polymerase III after the modifications mentioned in A, and the putative product of *Halobacterium* ORF gene (10). The regions of identity between the three sequences are boxed. Note that the *Drosophila* sequence consists of incomplete open reading frame, lacking the putative N-terminal 94 residues. The putative GTP-interacting regions (G-1 to G-5) are shown by large dotted line above the amino acid sequence.

DRG protein sequence contains all these five regions with their essential features conserved (Figure 3). Interestingly, these particular regions are identical among DRG and its closest homologues, the *Drosophila* and *Halobacterium* sequences mentioned above (Figure 2A),

	G-1		G-2		G-3		G-4		G-5	
CONSENSUS		xooooGxxxoGKBxL		ZxxTx		oxooDxxGxx		ooVoNKxD		SAx
EF-Tu	13	nvgtiGhvdhGKTtL	58	RgiTi	76	yahvDcpGha	131	ivflNKcD	173	SAl
H/K/N-RAS	5	klvvvGaggvGKSaL	32	YdpTi	53	ldilDtaGqe	112	vlVgNKcD	145	SAk
RHOA	7	klvivGdgacGKTcL	34	YvpTv	55	lalwDtaGqe	113	ilVgNKkD	160	SAk
RAB1	13	kllliGdsgvGKScL	40	YisTi	66	lqiwDtaGqe	120	llVgNKcD	154	SAk
Gs	42	rllllGagesGKSti	201	RvlTs	219	fhmfDvgGqr	288	ilflNKqD		
Spo0B	160	dvglvGfpsvGKStL	188	YhfTT	208	fvmaDlpGli	278	iiVaNKmD	310	SAv
DRG	66	rigfvGfpsvGKStL	94	YefTT	113	iqllDlpGii	244	iyVlNKiD	271	SAh

Figure 3. Comparison of amino acid sequences in the conserved GTP-interacting domains derived from the members of different classes of GTP-binding proteins. The domains G-1 to G-5 are as described (11). The consensus sequence is derived from the homologies between the members of different classes of GTP-binding proteins. x, denotes any amino acid; o, a hydrophobic residue; and Z, an arginine (R) or tyrosine (Y) residue. The sources of published sequences are as follows: EF-Tu (17); H-RAS (18); K-RAS (19); N-RAS (20); RHO A (21); RAB1 (21); Gs (22); and Spo0B (12).

suggesting their functional importance. *Drosophila* G-1 sequence derived from the cDNA sequence (G. Petersen, K. Sommer, and E.K.F. Bautz, personal communication), not shown in the figure, is also identical to that of DRG and the *Halobacterium* putative proteins. Outside the regions G1- G5, there were little similarities between DRG and other known GTP-binding proteins except for *Bacillus subtilis* Spo0B protein (12). This protein showed 37% identity in 127 amino acid residues towards the NH_2-terminus of DRG (data not shown). The limited homology of the DRG protein with other known GTP-binding proteins, outside the G1- G5 domains, makes it a novel GTP-binding protein of a new subclass. It is also noteworthy that the DRG and its closest *Drosophila* and *Halobacterium* homologues, lack the COOH-terminal motifs, -C-A-A-X, -C-C or -C-X-C (A, nonpolar residue; X, any residue) responsible for membrane anchoring of several low molecular weight GTP-binding proteins (13,14).

DRG Binds GTP

To study the biochemical properties of DRG protein, we expressed full length DRG fused with either MBP (4,5) or GST (6). The fusion proteins were purified from the bacterial cell extracts by affinity chromatography. The MBP-DRG fusion protein was used to raise a polyclonal antiserum in rabbits. Preliminary experiments showed that the antiserum could detect the DRG fusion proteins in immuno blot analyses and could immunoprecipitate a rabbit reticulosite lysate-synthesized protein translated from RNA prepared *in vitro* using T3 and T7 RNA polymerases from a *drg* cDNA template cloned into pBluescript (data not shown). To check the GTP-binding activity of the DRG protein, we incubated the bacterially expressed GST-DRG fusion protein or an extract prepared from E13 brain with radiolabeled GTP, UV-irradiated the reaction mixtures, immunoprecipitated using anti MBP-DRG serum and analysed the immunoprecipitates on SDS-polyacrylamide gels (Figure 4). Both GST-DRG and an E13 brain protein with a relative molecular weight of 40.5kD reactive with the antiserum were found to bind GTP while GST alone did not (Figure 4). The MBP-DRG fusion protein also binds GTP (data not shown). Both fusion proteins seemed to bind GTP relatively poorly when compared to the protein detected in E13 brain extracts and we speculate that the MBP/ GST moieties of the fusion proteins hinder the

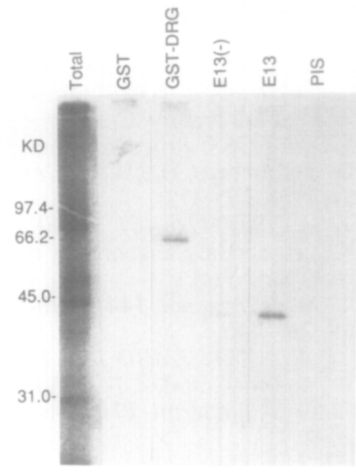

Figure 4. GTP-binding activity of DRG.
Purified GST, GST-DRG fusion protein (1μg each), or E13 brain extracts (10μg), UV-crosslinked with 10 μCi of [^{32}P] GTP, immunoprecipitated with anti MBP-DRG serum, electrophoresed on SDS/ polyacrylamide gels, and analysed by autoradiography.
In the first lane (Total), 1/50 th volume of an E13- [^{32}P] GTP crosslinking reaction, prior to immunoprecipitation was electrophoresed. (-) indicates that Mg^{++} was omitted from the E13 crosslinking reaction. PIS, preimmune serum was used for immunoprecipitation of E13- [^{32}P] GTP crosslinked reaction.

interaction by masking the GTP-binding domains of DRG. Judged from its size and immunoreactivity the 40.5kD protein found in the E13 brain extracts most likely represents the endogenous DRG protein. So far our attempts to show GTP hydrolysis by DRG fusion proteins, have resulted in variable results. Under the conditions where bacterially expressed Ras protein can hydrolyse GTP at substantial rates, MBP-DRG fails to show any activity while GST-DRG shows low and variable activity (data not shown). This could be attributed to the use of fusion proteins having inappropriate conformation or to the use of assay conditions inappropriate for this novel protein.

In summary, we have cloned the cDNA for a novel GTP-binding protein which shows remarkable degree of interspecies conservation. The *drg* gene and protein also show interesting pattern of expression during murine embryonic development (see following paper). As GTP-binding proteins are known to be involved in diversed cellular functions (15,16), our observations would suggest that *drg* may also play an important role in cell physiology.

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